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(54) METHOD AND KIT FOR THE SEPARATION, CONCENTRATION AND ANALYSIS OF CELLS

METHODEN UND MATERIAL FÜR DIE TRENNUNG, KONZENTRIERUNG UND ANALYSE VON ZELLEN

PROCEDE ET MATERIEL DE SEPARATION, CONCENTRATION ET ANALYSE DE CELLULES

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Description

FIELD OF THE INVENTION

The present invention is generally directed to separating and concentrating cells from non-cellular components in a milk sample, such as raw milk, pasteurized milk or reconstituted powdered milk, or in cultures of microorganisms from other types of food, such as meat, or other sources, such as stool or blood, and then analyzing the separated cells, as for the presence of undesireable microorganisms.

BACKGROUND OF THE INVENTION

Procedures for counting or quantifying cells in milk samples fall into one of two categories. In the first category, bovine somatic cells in raw milk samples are counted by various means to identify milk producing animals that may have bovine mastitis, an undesirable condition which limits the quality and quantity of milk production in infected animals. Mastitis testing procedures include direct cell counting using automated instruments and bioluminescent somatic cell ATP determinations following cell lysis with agents such as detergents that release cellular adenosine triphosphate (ATP). The number of somatic cells originally present in the sample is estimated from the measurement of the ATP released.

In the second category, cells of simple, usually unicellular microorganisms, such as fungi and bacteria, referred to hereinafter collectively as "microorganism cells," are counted in milk samples using various counting procedures. These procedures are generally used in the assessment of milk quality, particularly to screen out grossly contaminated milk samples.

Of the procedures used for microorganism detection, the Breed Smear (Breed, R.S., Zbl. Bakt. IIte. Abr. 30:337, 1911) is generally the quickest method. In this technique, a milk sample is smeared onto a slide, dried, stained, and the bacteria are counted using microscopic examination. The drawbacks of this procedure are that both viable and non-viable organisms are counted, and if milk samples contain fewer than 10^5 organisms/ml, many fields in the microscope must be counted to obtain statistically valid results. Microscopic evaluations are tedious and lead to operator fatigue.

The most widely utilized milk microorganism detection method is the standard plate method, which utilizes direct colony counting after plating in or on a growth medium. Standard methods (Standard Methods for the Examination of Dairy Products, 15th Ed., 1985, Richardson, G.H., Ed., American Public Health Org. Washington, D.C.) have been developed for milk samples, and many laboratories evaluate milk samples using either manual or automated plating procedures. While these methods have been utilized worldwide, there are certain disadvantages in using them. First, in the manual plating methods, two or more dilutions of the milk sample must be plated so that statistically significant plate numbers may be obtained. Second, plates for both the manual and automated plating procedures are usually incubated at elevated temperatures (e.g., in the US, 35°C; in Japan, 32°C); and, at these temperatures, the growth of psychrophillic organisms is repressed, yielding artificially low plate counting numbers. Finally, incubation periods of about 48 hours are required before bacterial plates can be counted and still longer plating periods are necessary for fungi. During this incubation period, bacterial numbers are increasing in the bulk milk from which the sample was taken, so that the result obtained is an underestimate of the actual number of colony forming organisms in the milk after the test. The delay in processing the raw milk to accommodate this incubation period by itself lowers the quality of the raw milk, and can contribute to shorter shelf lifetimes of the final product.

Plating or colony counting methods can be either manual or automated. Manual methods include the plate loop method (Thompson, D. I. Journal of Milk and Food Technology 30, p. 273, 1967) and the Standard Plate Count (supra).

Semi-automated or automated colony counting methods include the Spiral Plating Method (Gilchrist, J.E. Appl. Micro. 25, p. 244, 1973) and methods carried out by electronic colony counters (Fleming, M.G. Ir. J. Agric. Res. 14, p. 21, 1975). The Direct Epi-Fluorescent Technique (DEFT) is a fluorescence-labeling technique which can be performed manually or with automatic instrumentation. Other techniques include impedance measurements after growth of milk organisms and radiometric procedures utilizing radioactive glucose.

Another approach to microbe evaluation has been the utilization of the bioluminescent measurement of ATP from living cells in milk using firefly luciferase (Lumac® bv, The Netherlands). In this scheme somatic bovine cells in raw milk are lysed with a detergent, which releases somatic cell ATP. This ATP from bovine cells, and any other non-microbial milk ATP, is degraded with an ATPase, usually potato apyrase. Finally, a bacterial lysing agent is added and bacterial ATP is measured in a bioluminescence assay using firefly luciferase. While much data has accumulated in the literature on these methods, the sensitivity has been inadequate for routine milk testing due to milk and extractant inhibition of the luciferase reaction, incomplete removal of non-bacterial ATP, deleterious effects of apyrase on bacterial ATP detection (Theron, D.N., J. Food Prod. 49:4-7, 1986) both before and after bacterial cell lysis, and inefficient extraction of bacterial ATP. Literature data (Webster, J. A.J., Hall, M.S., Rich, C.N., Gilliliar, S.E., For, S.R., Leach, F.

R., J. Food Prod. 51: 949-954, 1988) for this type of assay suggest a cell sensitivity of approximately 1 x 10^6 cells/ml, which cannot be utilized in the United States where a cutoff for acceptance of 1 x 10^5 cells/ml for Grade A raw milk is required.

A logical improvement of this method that has been tried is concentrating the milk bacteria prior to the ATP assay. Various techniques have appeared in the literature which make use of cell filtration concentration (Peterkin, P.I., Sharpe, A.N., Appl. Environ. Microbiol., 39: 1138-1143, 1980), although these techniques are quite cumbersome, slow, and still exhibit most of the technical problems discussed above.

While all of the techniques mentioned above have demonstrated some measure of success, none has proven to be inexpensive, simple, accurate, fast and sensitive enough to provide the milk industry with a type of test which can be used satisfactorily for routine testing.

Methods of assaying bacterial cultures grown from food (including milk and meat) samples for Salmonella contamination using an immunoassay technique, employing antibodies to an antigen common to Salmonella spp., and a nucleic acid probe hybridization technique, employing DNA probes for Salmonella DNA, are available. These methods, however, require cumbersome and time-consuming growth of bacterial cultures, from the food material being analzed, before the assays for Salmonella can be carried out.

The need for such culturing prior to application of nucleic acid probe hybridization techniques to detect contamination, by undesireable microorganisms, of food materials, including milk, meat (e.g., chicken, turkey, beef, pork, horse, goat, whale and the like), eggs, fish, mussels, molluscs, crustaceans, vegetables, fruits, grains, and the like can be avoided, or the time for culture significantly reduced, if nucleic acid amplification techniques, such as the well known polymerase chain reaction (PCR) technique, are employed to increase the concentration of nucleic acid segments, characteristic of microorganism contaminants, to levels that are readily detectable by nucleic acid probe hybridization or nucleic acid staining methods. However, target nucleic acid amplification techniques have not been successfully applied for this purpose with cultures or extracts of specimens of food materials, or other materials of biological origin, such as blood, urine or stool, unless, prior to application of processes for amplification, microorganisms from the specimens have been subjected to cumbersome, costly, and otherwise undesirable, special treatments, such as with proteolytic enzymes, high concentrations of guanidinium salts, and detergent, or boiling, and the DNA from the microorganisms has been processed by similarly undesirable procedures such as ethanol-precipitation or chromatographic separation with or without phenol/chloroform extraction. These treatments have been regarded as necessary to separate nucleic acid, to be subjected to amplification, from contaminants, that interfere with the enzymatic reactions necessary for the amplification and that are thought to be provided by the microorganisms themselves or otherwise provided from the specimens to the microorganisms separated therefrom. See, e.g., Hill et al., Appl. Environ. Microbiol. 57: 707-711 (1991); Keasler and Hill, Abstracts of the 91st General Meeting of the American Society for Microbiology. Abstract No. P-12, p. 269 (1991); Olive, J. Clin. Microbiol. 27: 261-265 (1989).

SUMMARY OF THE INVENTION

According to a first aspect of the present invention, a method of separating and concentrating cells from an aliquot of a culture or extract of a material of biological origin, comprises the steps of:

(a) combining said aliquot with an aqueous suspension of a microparticulate carrier, to form a clearing solution; and
(b) separating the cells from the clearing solution. Such a method can be carried out in a simple and reliable manner.

The present invention can be used to analyze various types of liquid milk products, including raw milk, pasteurized milk, reconstituted dry milk, cream, ice cream and other milk products and derivatives.

In one embodiment, such a method comprises adding a chelating agent to a milk sample, and then separating the cellular components from other milk components, such as by centrifuging the sample combined with chelating agent for a brief period of time, and separating or decanting non-cellular milk components from the separated cellular pellet. An agent, such as a detergent, to lyse animal cells but not microorganism cells of interest, may be included in the sample during the centrifugation.

The components of the cellular pellet resulting from such separation are amenable to a variety of analyses, with such analyses being free from interferences that would be caused by contaminating milk components. The cellular pellet may contain both somatic cells (i.e., mammalian cells from the mammal that is the source of the milk) and microorganism cells which can then be microscopically examined to determine the relative concentration of each. The somatic cells may also be removed by adding a lysing agent, such as a detergent, to the sample with the chelating agent in the clearing solution, with an agent being chosen which lyses only the somatic cells. The pellet remaining after centrifugation will thus contain almost exclusively microbial (i.e., microorganism) cells, which can be analyzed in various ways, including analysis for ATP concentration after extracting ATP such as by lysing of the microorganisms. Filtration may also be utilized to separate cellular components from other milk components in the milk sample to which

the clearing solution has been added.

The presence of animal cells, such as bovine somatic cells, in a raw milk sample, can also be determined in a quick and simple manner. The invention is further suited to the analysis of contamination of milk samples by a variety of microorganisms such as bacteria, yeasts and molds, and spores from bacteria, yeasts and molds.

The concentrated cellular materials isolated from milk by way of the present invention may be utilised for various types of further analyses, including microscopic examination with dyes or stains, or with chromogenic, fluorescent, chemiluminescent or other detectable reporter molecules. The isolated concentrated cellular materials may also be used for plating, for either broad spectrum or specific plating of microorganisms, or for preparation of samples for any of the milk testing methods routinely utilized.

The beetle luciferase ATP determination method (DeLuca, M.A., Advances in Enzymology, Meister, A., editor, 44, 37-68, 1976) may be utilized in the present invention for quantification of cellular numbers in the original liquid milk sample. This ATP determination method is applicable to the quantification of either animal or microrganism cell numbers. The separated, isolated cellular material may be utilized in any type of enzymatic, immunological, or molecular biological testing method that quantifies or identifies specific organism types in a liquid milk sample. Among these methods are nucleic acid probe hybridization assays, including such assays following target nucleic acid amplification by PCR or other techniques.

The method of the present invention may also be used as a pretreatment method for a variety of samples for centrifugation or filtration on various types of filtration matrices such as membranes, hollow fibers or centrifugation type filters.

While chelating agent, detergent (lysing agent), and microparticulate carrier each contribute important, independent advantages in the separation of cells from milk samples, in the case of other food samples the combination of microparticulate carrier with either centrifugation or filtration is sufficient.

Surprisingly, microrganism cells from milk samples, or from cultures or extracts of prepared from samples of other food materials, as indicated above, or non-food materials of biological origin, such as blood, urine and stool, after separation from non-cellular components using a chelating agent, microparticulate carrier/clearing solution (in the case of milk) or a microparticulate carrier/clearing solution (in the case of other materials) and separation method in accordance with the invention, are free of contaminants, other than those present in the separated cells themselves, that inhibit enzymatic reactions that are necessary for target nucleic acid amplification. Thus, advantageously, the separated cells can be used directly, without need for isolation of nucleic acid from other cellular constituents, to provide nucleic acid for amplification. The cells simply need to be treated (as by being held briefly at an elevated temperature) to cause them to release nucleic acid, and to denature enzymes or other components that would degrade amplified nucleic acid or inhibit enzymatic reactions required for the amplification method employed. If the amplification method relies on an enzyme that would be irreversibly inactivated at the elevated temperature, the cells can be added directly to a solution of reagents required for amplification, other than the temperature-sensitive enzyme(s), the solution can be heated to the elevated temperature and then cooled, and the enzymes can be added to initiate amplification (assuming target nucleic acid is present). In a preferred method, only thermally stable enzymes will be used in the amplification process (preferably PCR). Then separated cells are added directly to a solution with all reagents, including enzymes, required for amplification, and the solution is heated to an elevated temperature (low enough so that the enzymes for amplification remain active), and then, when the temperature is reduced sufficiently for primers to hybridize to template, amplification begins. Target nucleic acid segment from the microorganisms sought to be detected, if present, will be amplified in the amplification process. The nucleic acid resulting from the amplification process can then be assayed, using, for example, any nucleic acid probe hybridization assay method, for target nucleic acid segment.

Thus, according to a second aspect of the present invention, a method for detecting the presence of cells having nucleic acid that comprises a preselected target segment, in a culture or in an extract of a material of biological origin, comprises obtaining a pellet of cells from the culture or extract by using a method according to the first aspect of the present invention (sometimes referred to as cell wash methods) and using therein centrifugation, provided that, if said culture of a material of biological origin is a liquid milk sample, said clearing solution further comprises a chelating agent; suspending cells from the pellet so obtained in a first solution and treating the first solution to provide a second solution of nucleic acid from the cells substantially without isolation of nucleic acids of the cells from other constituents of the cells; subjecting the nucleic acid of the second solution to a nucleic acid amplification process to provide a predetermined, amplified nucleic acid segment only if the preselected target segment is present in said cells suspended from said pellet; and assaying nucleic acid after the amplification process for the presence of said predetermined, amplified segment.

The cells of the pellet yielded by the cell wash methods may be simply washed prior to being suspended in the solution for treatment to provide nucleic acid for amplification. Preferably the assay, which is the final step of the method, will be a nucleic acid probe hybridization assay, which, as the skilled will understand, will employ a probe (preferably an oligonucleotide and labelled to be detectable) capable of hybridizing to amplified target segment. As the skilled will understand, by carrying out the method in parallel with appropriate standards, the method can be used not only to

detect the presence of preselected, contaminating microorganisms (if present at a level above the sensitivity of the method) in the material being tested with, but also to quantify the extent of contamination of the material with such microorganisms. An elevated temperature, which can be used to release nucleic acid from cells and denature or inactivate amplification-inhibiting substances from the cells, is above about 70 °C, and more preferably above about 90 °C, for analysis of samples of cells from milk, or cultures prepared from meat, eggs, or aquatic species used as food sources.

In preferred amplification processes, aliquots of solution with reagents (e.g., enzyme) will not need to be added over the course of the amplification process. A particularly preferred amplification method is the PCR method using a thermally stable DNA polymerase from a bacterium, such as Thermus aquaticus, which lives at high temperatures; in the particularly preferred method, thermal cycling will occur but the thermally stable polymerase will retain sufficient activity, notwithstanding the high temperatures reached in the cycling, that enzyme will not need to be replenished during the amplification process.

The present invention also encompasses as further aspects thereof, a clearing solution as defined in claim 25, and novel kits, as defined in claims 28, 30, 32 and 34, for use in carrying out the methods of the invention. Such kits include a clearing solution, which contains at least a chelating agent, a microparticulate carrier and a somatic cell lysing agent. The kits also may contain a solution for washing the cell pellet, and, for ATP detection, a microbial cell ATP extracting agent or lysing agent, a buffer solution and an ATP detection reagent such as luciferase-luciferin. Kits may also include components for preparing a sample for the Breed Smear test or other types of direct microbiological examination procedures. Kits of the invention may also comprise enzymes, buffers, and other reagents for nucleic acid amplification or detection of amplified nucleic acid in a nucleic acid probe hybridization assay or by staining.

DETAILED DESCRIPTION OF THE INVENTION

Further objects, features and advantages of the present invention will be apparent from the following detailed description when taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

In the drawings:

Fig. 1 illustrates the steps in the general method of the present invention using a liquid milk sample.

Fig.2 is a graph showing the correlation between CFU and RLU for the separation of microbial cells from milk described in Example 1.

Fig.3 is a graph showing the correlation between CFU and RLU for the separation of microbial cells from milk described in Example 2.

Definitions:

The term "bacteria" is meant to include single-celled prokaryotes. It is also within the scope of the present invention to interchange the terms "microbe" or "microorganism cell".

The term "eukaryotic cells" is intended to denote organisms in which the genetic material is enclosed by a nucleus.

The term "liquid milk sample" is meant to include all liquid solutions of origin from dairy raw materials or products including raw milk, ultra high temperature pasteurized milk, low temperature long time pasteurized milk, reconstituted powdered milk, cream, skim milk, liquefied ice cream or ice milk or related products, and suspensions of milk or dairy products in liquid samples. While bovine milk is the preferred material for application of the present invention, the invention is applicable as well to milk from any mammal.

The term "chelator" or "chelating agent" is meant to include all molecules or macromolecules that bind to or combine with calcium ions and may also bind with other divalent metal ions including magnesium ion, iron ion, zinc ion, cadmium ion, beryllium ion, cobalt ion, nickel ion, copper ion, lead ion, and other metal ions. The term "chelator" or "chelating agent" includes all synthetic and natural organic compounds known to bind these ions, and any molecule of biological origin, or by-product or modified product of a molecule of biological origin, such as proteins, sugars or carbohydrates, lipids and nucleic acids, and any combination thereof, that may bind the above mentioned types of ions. The term "chelator" or "chelating agents" also includes any solid material of naturally occurring or synthetic origin that binds calcium and to a lesser extent magnesium and perhaps one or more of the other above-mentioned ions.

While various procedures utilized in the overall invention are generally known to the art, the combination of these procedures in accordance with the invention has not been contemplated. General methods known to the art, which play a part of the overall assay techniques of the present invention, include the standard plating techniques of microorganisms from dairy products, staining and identification methods for microorganisms, bacterial extraction methods, the use of separated, concentrated cellular materials in other procedures, and general chelating chemistry. A discussion

of one or more of the above-noted techniques can be found in the following references, which are incorporated herein by reference: Standard Methods for the Examination of Dairy Products, 15th Ed., 1985, Richardson, G. H., Ed.; American Public Health Assoc., Washington, D.C.; Bacteriological Analytical Manual, 6th Ed., USDA, 1984, Marcel Dekker Inc., New York; Sambrok J., Fritsch, E.F., and Maniatis, T., Molecular Cloning, 2nd Ed., Ferguson, M., Ed., Cold Spring Harbor Laboratory Press, 1989; O'Connor, F., Australian J. Dairy Tech., June 1984, pp. 61-65, 1984 (this reference includes descriptions of microbiological milk testing methods and relevant references for each method) ; Microbiological Reviews 44, 739-769, Karl, D. M., 1980; Martell, A.E., Chemistry of the Metal Chelate Compounds, Prentice-Hall, New York, 1952; Current Protocols in Molecular Biology, John Wiley & Sons, New York, New York, 2 volumes (supplemented) (1987 - 1991).

As indicated in the cited Molecular Cloning and Current Protocols in Molecular Biology, nucleic acid amplification techniques, particularly those with thermally stable enzymes, such as PCR amplification employing DNA polymerases from Thermus aquaticus, are well known, as are nucleic acid probe hybridization assay methods and staining methods for detecting nucleic acid segments. Additional information on such techniques can be found in United States Patent Nos. 4,693,195 and 4,693,202 (PCR and its application in nucleic acid probe hybridization assays for diagnosis); International Patent Application Publication No. WO 88/10315 (transcription-based nucleic acid amplification/ detection methods); United States Patent No. 4,889,818 and International Patent Application No. PCT\US90\04169 (published February, 1991)(thermostable DNA polymerases from Thermus aquaticus (Tag DNA polymerases) and their use in PCR amplification); European Patent Application Publication No. 0 329 822 (isothermal transcription-based nucleic acid amplification process); United States Patent No. 4,957,858 (Q-Beta replicase catalyzed autocatalytic replication of replicatable RNA linked to probes complementary to target segment).

The present invention encompasses a separation and concentration technique for the removal of cellular materials from liquid milk samples and other cultures. While the technique will now be described with reference to a liquid milk sample, it will be understood that it may be applied as well to cultures prepared from other materials.

In carrying out the technique, a milk sample is placed in an appropriately sized centrifugation vessel as shown at 10 in Fig. 1 and a chelating agent and a microparticulate carrier, and optionally a lysing agent, are added. The chelating agent may be one of various types as described above, which by way of illustration only, may include ethylenediamine tetraacetic acid (EDTA, Versene®,), bis-(o-aminophenoxy) -ethane-N,N,N^1,N^1-tetra-acetic acid (BAPTA), ethyleneglycol-bis-(β-aminoethyl ether) N,N,N^1,N^1-tetraacetic acid (EGTA), nitrilotriacetic acid (triglycine, ammonia triacetate, Triton A), trans-1,2-diaminocyclohexanetetraacetic acid (CDTA) , diethylenetriaminopentaacetic acid (DTPA), citrate, arginine, hypoxanthine, 4,5-dihydroxybenzene-1,3 -disulfonic acid, sodium phosphate glass or any of the molecules in the "glass" or polyphosphate family, crown ether type compounds and all derivatives and precursors of such molecules. The chelating agent, for example EDTA, and carrier or lysing agent, if present, are preferably added to the milk sample together in a solution, called a "clearing solution"; and the mixture of chelating agent, and carrier or lysing agent, and milk sample is capped and inverted or vortexed to mix. The sample is then placed into a centrifuge of appropriate size, and the sample centrifuged at 10,000 x g (minimum relative centrifugal force) for a minimum of 5 minutes. After centrifugation the sample separates into three distinct phases. The uppermost phase is a cream and milk protein "pad" illustrated at 15 in Fig. 1, and this pad floats at the very top of the liquid sample. Beneath the pad is the second "clear" liquid zone, illustrated at 16 in Fig. 1, which is, unlike a milk sample, non-opaque and translucent. Finally, at the bottom of the centrifuge tube is the cellular pellet 17, the size of which is dependent on the number of cells in the original milk sample and the type of chelating agent(s) and/or microparticulate carrier and/or detergent(s) used in the treatment. The pellet may also contain a small amount of other milk components which are associated with the cells. After clearing of a typical 1.0 ml raw milk sample, the pellet is approximately 10 to 40 µl in volume and is white or off-white in appearance.

The proposed effect of the chelating agent in the present invention is the dissociation of casein micelles in a milk sample into "sub-micelles" (L.C. Chaplin, J. Dairy Res. 51: 251-257, 1984). After the chelating treatment, micellular milk protein material rises to the top of a centrifuge tube or remains in solution, as opposed to pelleting in the absence of chelator. The chelator binds calcium ion which is a major component that contributes to micelle structure (S.H.C. Lia, Biochemistry 11:1818-1821, 1972). Thus, chelating agents that bind calcium ion particularly well are preferred in the present invention.

In the separation method of the invention, it is of great importance that the chelating agent act on the milk micelles while not negatively affecting the cells to be studied. For example, a chelating agent that may clear milk but also removes, lowers or diminishes microbial cell ATP or viability, would be a poor candidate for an assay method, such as one using beetle luciferase, that measures microbial ATP. For this reason certain chelating agents are superior to others for certain types of applications. Chelating agents which have been found particularly suitable are nitrilotriacetic acid and ethylenediamine tetraacetic acid.

The present invention employs microparticulate carrier for the collection of cells, especially microbial cells, during the centrifugation steps of the cell separation process. The microparticulate carrier serves to assist in the pelleting process. The physical nature of the microparticulate carrier is such that the carrier sediments, or pellets, slightly slower

than the microbial cells and as such makes the cell collection more quantitative.

A number of materials could be used as microparticulate carriers, including "beads" of polystyrene, latex, plastic, glass, diatomaceous earth, metal oxides, and colloidal materials including polyacrylamide, dextrans, cross-linked dextrans, and starches. The properties of the microparticulate carrier that are desirable are threefold: 1) The carrier should sediment as fast as, or preferably slightly slower than, the cells of interest that are being collected. This characteristic is basically a function of the particles' density, size and charge. 2) The carrier should be amendable to resuspension after centrifugation for the purpose of facilitating treating or evaluating the cell pellet. 3) The carrier must be inert to the testing or evaluation that will be performed on the cell pellet. For example, the carrier should be invisible in cell-staining techniques if a microscopic examination is to be performed, or the carrier should not bind an analyte that will be measured, such as ATP for the measurement or estimation of microbial cells. Beads of materials cited above with diameters between about 0.25 µm and 2.5 µm are suitable as microparticulate carriers. Surfactant-free polystyrene beads with a diameter of about 1 µm are the preferred microparticle carriers.

The use of microcarrier particles in the cell separation method of the invention enhances the ease with which an operator is able to remove unwanted supernatants from cell pellets following a centrifugation. The carrier serves as a visual indicator much larger than the microbial cell pellet and as such makes the removal of the supernatants much more convenient. In addition, the probability of mistakingly aspirating part or all of the cell pellet is greatly reduced when a microparticle carrier is used.

If one wishes to remove somatic cells from a raw milk sample and separate out and concentrate only microbial cells, a non-ionic detergent such as Triton X-100 or Nonidet P-40 (NP-40) may be added to the milk sample in combination with the chelating agent. Such a detergent will specifically lyse bovine somatic cells (animal cells) without lysing microbial cells. Because the somatic cells are lysed in the treatment prior to centrifugation, there will be essentially no intact somatic cells in the post-centrifugation pellet. Subsequent ATP determinations made on these pellets will thus detect only the bacterial cells or other microbe cells.

If, on the other hand, one wishes to measure or quantify the number of somatic cells in a milk sample, a detergent identical to that mentioned above can be added to the cellular pellet and only somatic cells will be lysed. A cellular metabolite such as ATP which was contained in the somatic cells can then easily be measured without any elevation of the result by microbial ATP, which cannot be extracted with the detergent. The number of somatic cells that were present in the sample can then be calculated using the known amount of ATP that was measured and the average amount of ATP known to exist in somatic cells. This procedure is an improvement over previously published methods (R. Bossuyt, Milchwissenschaft 33:11-13, 1978).

The present method provides a useful procedure for concentrating cells and eliminating background milk contaminants such as casein and casein micelles, lipophilic components, and salts. To perform this concentration, milk, for example, one ml, is cleared by centrifugation with a chelating agent, and the resulting pellet is resuspended in a very small volume (for example 10 µl) of the appropriate buffer or liquid. In this example, all of the cell components are removed from the milk contaminants and are concentrated 100 fold. This concentrated sample can then be utilized in other analyses as desired.

One example of the usefulness of this procedure is in staining and counting microorganisms from raw milk. If a milk sample contains less than 1×10^5 organisms per ml, and 10 µl of milk is put onto a microscope slide and stained, many fields of the stained specimen must be viewed in order that statistically significant counting data be collected. The fields are difficult to score because of background staining of other milk components. This procedure, the Breed Smear procedure, is widely used, particularly in Japan, for raw milk quality assessment. By utilizing the foregoing concentration step (100 fold concentration) many fewer fields need to be counted and fields are much easier to score because the background is much clearer. This speeds up specimen throughput and reduces operator fatigue and error.

Alternatively, somatic cell numbers in a specimen may be quantitated by separating all cells from the various other components of milk and concentrating the cells in accordance with the present invention.

Microbial cells in a pellet which is free from contaminating somatic cells can be lysed with a microbial extracting agent and assayed for ATP. The number of microbial cells is then estimated using the ATP measurement and the average amount of ATP known to exist in microbial cells, or more specifically in milk microbial cells. This type of procedure offers a number of advantages over other methods such as standard plating and spiral plating. First, it is much faster, because a result is available in as little as one hour as opposed to the 48 hour time frame for the other two methods. Second, the ATP measurement will give results that are not influenced by cell clumping, i.e., ATP will be measured from all cells; in plating methods cell pairs or groups of cells are scored as a single cell because one colony is formed. Third, the ATP method will measure ATP from psychrophillic organisms which will be missed in plate counting methods that culture at temperatures over 30°C.

As mentioned before, cells which are separated and concentrated using the present invention can be subjected to various other methodologies with the background greatly reduced and the cell number (and associated signal) greatly increased due to concentration. This provides the possibility of greater sensitivity, and more reliable, reproducible results due to background elimination.

A method of the present invention may also include cell enrichment or enhancement techniques either before or after the clearing centrifugation so that sensitivity, cell number, or cell hardiness may be improved. For example, by treating cells with treatments (D.P. Theron, J. Food Prot. 46:196-198, 1983) or components (K. M. Oxley, Bioluminescence and Chemiluminescence, Ed. J. Scholmerich, John Wiley & Sons, N.Y., pp. 495-198, 1986) that increase cellular ATP, the sensitivity of the procedure may be enhanced. This type of procedure can also be applied to detecting specific types of cells in a particular sample, using selective media or even polyclonal or monoclonal antibodies.

Persons of skill in the art are well aware of the need for, and nature of, appropriate controls in assay procedures of the type employed in the present invention and appropriate standards to allow quantitative information on analyte concentration or quantity to be determined from such assay procedures. Such controls and standards are illustrated in the following Examples.

Reference is now made to Fig. 1, where the clearing wash method of the present invention is illustrated with reference to a milk sample. In this procedure, a milk sample is first placed into a centrifugation vessel 10. Next, a chelating agent, plus or minus microparticle carrier and plus or minus a detergent, is added using a clearing solution, as indicated. The contents of the vessel are mixed, the vessel is then centrifuged, and the resulting sample is said to be "cleared". If a milk sample without a chelating agent were to be centrifuged in the same manner, there would be no "clearing" but a large, diffuse, milk protein and cell pellet at the bottom of the tube. Finally, the cell pellet is freed from most of the other materials in the vessel by aspiration of the supernatant after the centrifugation.

Kits for the analysis of cellular components from liquid milk samples can be formed of the following components:

A microbial test kit using ATP detection comprises:

(a) A clearing solution containing a chelating agent, a somatic cell lysis detergent such as the chelating agents and detergents described above, and a microparticulate carrier.
(b) Optionally, a solution for washing the cell pellet.
(c) An ATP extractant such as a cell lysis solution, as described above, for releasing microbial cell ATP.
(d) Optionally, a buffer solution.
(e) An ATP detection reagent, such as luciferase (e.g., from P. pyralis)-luciferin for measurement of ATP by bioluminescence.

A microbial test kit using the Breed Smear comprises:

(a) A clearing solution as described for the microbial test kit using detection of ATP.
(b) Optionally, a solution for washing the cell pellet.
(c) A solution for staining cells.

A bovine somatic cell test kit using ATP detection comprises:

(a) A clearing solution as described above for the microbial test kit using detection of ATP, excluding the somatic cell lysis detergent.
(b) A somatic cell lysis solution that does not lyse microbial cells.
(c) An ATP detection reagent such as luciferase-luciferin.

A microbial detection kit for detection of microorganisms via nucleic acid amplification and nucleic acid probe hybridization comprises:

(a) A clearing solution which comprises a microparticulate carrier and, in the case of a milk sample, will also comprise a chelating agent.
(b) Optionally, solution for washing the cell pellet from the cell wash method using the clearing solution.
(c) Enzymes and primers required for amplification of a target nucleic acid segment characteristic of the microorganisms to be detected. Optionally, other, more commonly available components used in the amplification, such as nucleoside triphosphates (2'-deoxyribonucleoside triphosphates in case only DNA is synthesized in the amplification process), buffers, and the like may be provided.
(d) Nucleic acid probe for target segment amplified with the components in (c) together with reagents required to detect probe hybridized to said target segment, if the probe per se is not detectable (e.g., as labelled with ^{32}P). For example, if the probe is labelled covalently (i.e., directly) with an enzyme, such as alkaline phosphatase, or non-covalently (i.e., indirectly), through biotin covalently linked to the probe, with an avidin-enzyme (e.g., alkaline phosphatase) complex, wherein the enzyme catalyzes production of a chromophore, which provides the detected signal, then reagent for production of the chromophore and, for indirect labeling, avidin-enzyme complex will be provided. Again, buffers, solid supports for hybridization, and the like may also be provided.

(e) Alternatively, or optionally in addition, to nucleic acid probe and associated reagents as in (d), a staining reagent such as ethidium bromide to detect amplified target of known size, as on a gel. Reagents for preparing a suitable gel, running in parallel a sizing gel, and the like may also be provided, as the skilled will understand.

Each of the foregoing kits may optionally include a filter, for example, as described in Example 6 below, and reagents to provide suitable controls or, for quantification, standards.

The invention is further illustrated by the following, non-limiting examples.

Reference Example 1

This example discloses the separation of microbial cells from an artificially innoculated milk sample. In addition, the assay is compared to a commercially available milk ATP assay to compare the relative sensitivities of the two procedures.

Raw milk samples were obtained from a local dairy and the raw milk was streaked on standard methods agar (Standard Methods for the Examination of Dairy Products, supra) to isolate individual colonies. Eleven visually different colony types were picked and grown in standard methods broth in an attempt to obtain a representative sample of the different species that may be found in raw milk. Of these isolates one cell type (Serratia liquefaciens) was chosen for the experiment.

A pasteurized milk sample was used as a negative control for the procedure. To one ml of this pasteurized milk was added 10 µl of the overnight grown (23°C), milk--isolated bacteria (S. liquefaciens). Using this artificially inoculated milk sample and uninoculated pasteurized milk, a number of inoculated milk samples were prepared as set forth in Table 1 below.

Reference Example 2

This example discloses the separation of microbial cells from eleven raw milk samples and one pasteurized milk sample.

Raw milk samples were received from a local dairy and stored at 4°C. A pasteurized milk sample was also included in the study. One ml of each milk sample was pipetted in duplicate into 1.5 ml Eppendorf tubes. The milk samples were allowed to incubate at room temperature for 10 minutes followed by the addition of 0.5 ml of 0.5% NP-40, 3% nitrilotriacetic acid (sodium salt) to each tube. The tubes were then capped and mixed by inverting 10 times.

The tubes were centrifuged at room temperature for 5 minutes at 12,000 x g and the supernatants aspirated as in Reference Example 1. To each pellet 0.5 ml of a 0.05 mM $MgCl_2$, C.2% NP-40 solution was added, the tubes were capped and vortexed, and centrifuged for 5 minutes as before. After centrifugation the supernatants were again aspirated, and the washing, centrifugation and aspiration were repeated one more time.

The pellets obtained were treated with TCA solution and read in a luminometer, as described above in Reference Example 1. For each sample, the result is determined as the mean of the duplicate measurements.

Each milk sample was also diluted with sterile 0.8% NaCl and 1.0 ml of 10-fold dilutions were pipetted into duplicate petri dishes. Approximately 20 ml of sterile standard methods agar was added to each dish and mixed. The plates were incubated at 23°C for 48 hours and colonies were then counted. Results are the means of duplicate plate counts.

Reference Example 3

This example discloses the separation of microbial cells from 65 raw milk samples using a modification of the procedure presented in Reference Example 2.

The milk samples were treated as in Reference Example 2, with an additional step of adding a protease treatment to treat the microbial pellet.

After the first centrifugation step the resulting pellet was dissolved in 500 µl 0.05mM $MgCl_2$, 0.2% NP-40, and 30µg/ml α-chymotrypsin (Sigma Chemical Co., St. Louis, Missouri, USA, Cat. No. C7762). The pellet was vortexed three times for 2 seconds and the tubes were incubated at room temperature for 20 minutes. The remainder of the procedure is identical to that described in Reference Example 2.

Example 1

This example discloses the separation of microbial cells from 88 raw milk samples using a modification of the procedure presented in Reference Example 3.

To 1.0 ml of each raw milk sample was added 500 µl of a solution of 3% nitrilotriacetic acid (sodium salt), 0.5% Triton X-100, and the samples were then treated as in Reference Example 3. After aspirating the supernatants from

the first centrifugation step, a solution of 0.05 mM $MgCl_2$, 0.2% Triton X-100 containing 0.01% surfactant-free polystyrene beads (0.984 µm, Bangs Labs, Carmel, Indiana, USA) and 60 µg/ml α-chymotrypsin was added. The polystryene beads centrifuge down with the cells. The samples were incubated, centrifuged, and aspirated as in Reference Example 3, and the pellets were resuspended in 0.05 mM $MgCl_2$, 0.2% Triton X-100, vortexed, and recentrifuged. The remainder of the procedure was performed as described in Reference Example 3. The results of this experiment are shown in Figure 2. A positive correlation between the two methods was obtained in this study.

Example 2

This example discloses the separation of microbial cells from 18 raw milk samples using a modification of the procedure described in Example 1.

In this study an equivalent amount of carrier polystyrene beads (described in Example 1) were added to the 3% nitrilotriacetic acid (sodium salt), 0.5% Triton X-100 solution for the first milk treatment step. Carrier was not added in any subsequent steps. In addition, α-chymotrypsin was used as a final concentration of 150 µm/ml in the first wash solution. The remainder of the procedure is as described in Example 1.

The results of this correlation study are shown in Figure 3, which shows a correlation between the two procedures.

Example 3

Salmonella typhimurium strain PB637, obtained from a hospital in New England, was grown overnight in a rich broth. The overnight culture was diluted to an OD_{600} of 0.1 and then grown to an OD_{600} of 0.9. The culture was serially ten-fold diluted in phosphate-buffered saline (PBS). An aliquot of each dilution was plated to determine the titer of viable bacteria. Five µl of each dilution was added to 0.995 ml of raw milk in microcentrifuge tubes. There were 7 dilutions plus one blank of PBS with no bacteria. Next 500 µl of the clearing solution (0.25M EDTA, 0.5% Triton X-100, 0.01% microparticulate carrier (surfactant-free polystyrene beads, see Example 1)) was added to each tube. The tubes were inverted ten times to mix thoroughly. The tubes were centrifuged for 5 minutes in a microcentrifuge. The cream layer was removed and the supernatant was aspirated. The cells of the pellet were then washed by resuspending the pellet in 1 ml of PBS with the use of a vortex mixer, followed by adding 500 µl of the resulting cell suspension to a microcentrifuge tube and centrifuging for 5 minutes. The supernatant was removed by aspiration and the resulting pellet was resuspended in 25 µl of distilled water.

Amplification of a segment of the Salmonella genome was used for detection of the Salmonella present in the raw milk sample. Oligonucleotides designated A86, a 28-base DNA, and B83, also a 28-base DNA, were used as the primers. The primers were mixed such that each primer was at a concentra-tion of 50 µM in sterile, distilled water.

A PCR reagent mix was made by mixing 180 µl of 10X Taq DNA Polymerase Buffer (500 mM KCl, 100mM Tris-HCl (pH 8,8 at 25°C), 15mM $MgCl_2$, 1% Triton X-100) (Promega Corp., Madison, Wisconsin, USA), 10.8 µl (45 units) of Taq DNA Polymerase (Promega Corp.), 180 µl of dNTP solution (2 mM of each of dATP, dGTP, dCTP and dTTP) to provide an initial concentration of 200 µM of each of the dNTP's in the PCR reaction, plus distilled water to a final volume of 1.8 ml. To a new microfuge tube was added 93 µl of the PCR reagent mix and 2 µl of the primer mix (100 pmoles of each primer). Next 5 µl of each bacterial suspension was added to the tube and the liquid was overlaid with 2 drops of mineral oil. The tubes were placed in a Perkin-Elmer Cetus DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, Connecticut, USA). There were no steps taken to lyse the bacteria or extract the DNA. This was accomplished during the PCR reactions. The Thermal Cycler conditions were set for 1 min at 94°C, then 1 min at 65°C, and finally 2.5 minutes at 72°C with 5 second autoextend. A total of 35 cycles of PCR were performed.

The PCR products were analyzed by agarose gel electrophoresis. A 1.5% agarose gel in TAE buffer was loaded with 5 µl of each PCR reaction product. The gel was electrophoresed for 1.5 hr at 70 V. The gel was stained with ethidium bromide. The lane from the milk sample with 250 cells/ml of raw milk showed a visible band of the expected fragment length. Dilutions with fewer cells and the PBS control tube (PBS with no cells added to milk) showed no bands on the gel.

The gel was denatured in 0.5M NaOH for 30 minutes at room temperature. It was then washed two times in 1M Tris-HCl for 15 minutes each wash. The gel was then blotted by overlaying the gel with a nylon membrane followed by 0.5 inches of Whatman 3MM paper and Saran wrap, weighted with two large books. After 2 hours, the nylon was washed for 10 minutes in 2 X SSC and UV crosslinked in a UV-Stratalinker 1800 (Stratagene, La Jolla, California, USA) in automatic mode. The gel was hybridized with a ^{32}P kinase-labelled, 24-base DNA, designated P47, which has the sequence of a segment of the Salmonella genome between the two primers, overnight at 62°C in 2X SSC, 20 mM sodium phosphate, 0.1% SDS, 10X Denhardt's solution, 10% dextran sulfate, and 0.1 mg/ml herring sperm DNA. The nylon was washed 2 times for 15 minutes each at 62°C in 2 X SSC with 0.1% SDS. The nylon was exposed to Kodak XAR film at -70 °C for 5.5 hours. All of the dilutions of bacteria into raw milk produced a PCR product band that hybridized with PM407 while the PBS control did not. The highest dilution had approximately 2.5 viable cells added to

the 1 ml of raw milk. Only 1/10th of this material was added to the PCR reaction, indicating that either the sample taken for the PCR reaction just happened to contain one cell or that the culture had some non-viable cells present. In either case, it is clear that the procedure is highly sensitive for detecting Salmonella cells in raw milk.

Example 4

An experiment was done to detect Salmonella typhimurium in beef steak. Five samples (25g) of beef steak were each added to 225 ml tetrathionate broth and stomached in a Stomacher Lab-Blender 400 (Tekmar Co., Cincinnati, Ohio, USA) with 0, 0.02, 2, 200, and 20,000 Salmonella cells/ml as determined by an initial standard plate count (SPC) of the inoculum done on bismuth sulfite agar plates. The samples were incubated at 37°C with shaking (150 rpms). At 0, 3 and 24 hours, a plate count and "clearing wash PCR assay" (an assay similar to that described in Example 3, beginning with the clear wash procedure to obtain the initial cell pellet) were performed on each of the five broths. The plate counts were also done on bismuth sulfite agar (selective for and indicative of the growth of the Salmonellae), since it was anticipated that the meat was precontaminated with bacterial flora. Table 2 provides results from the counts on bismuth sulfite agar.

Table 2

Bismuth Sulfite Plate Counts in Cells/ml # Salmonella Added per Milliliter					
	O	0.02	2	200	20,000
T=Oh	0	0	$1.2x10^2$	$7.0x10^2$	$3.5x10^4$
T=3h	0	0	$1.0x10^2$	$4.5x10^3$	$3.6x10^5$
T-24h	$9.5x10^6$	$1.2x10^9$	$1.6x10^9$	$1.8x10^9$	$1.6x10^9$

The clearing solution was as in Example 3 (0.25 M EDTA pH 8.0/0.5% Triton X-100/0.01% carrier). The pellets were resuspended in 100µl PBS and then quickly frozen at -70°C. After all the samples had been taken and prepared, they were thawed and vortexed. 5 µl were withdrawn from each and added to 95 µl of PCR reaction mix (see Example 7). PCR amplifica-tion was carried out using the Perkin-Elmer Cetus DNA Thermal Cycler with oligonucleotides designated C84 (a 27-base DNA) and A86 as primers. The primers bracket a Salmonella genomic segment, which includes a subsegment with the sequence of 24-base DNA P47. The PCR cycle consisted of 1 min at 95°, 1 min at 65°, and 2 min 30 sec at 72° with a 5 sec autoextend. A 1.5% agarose gel was run on the PCR products. The gel was then treated with NaOH, neutralized, and squash-blotted onto a nylon membrane. The membrane was rinsed and UV crosslinked before being probed with ^{32}P kinase-labelled P47 as probe. Subsequent autoradiography confirmed that the products were amplified Salmonella genomic DNA segments which included a subsegment with the sequence of P47. A positive signal was noted for the 20,000 cells per ml samples at 0 and 3 hours incubation and at all inoculation concentrations at 24 hours incubation time. Thus, the clearing wash method to concentrate cells following an overnight culture prepared from meat samples is a satisfactory method of sample preparation for PCR analysis for microorganism contamination.

Claims

1. A method of separating and concentrating cells from an aliquot of a culture or of an extract of a material of biological origin, comprising the steps of:

   (a) combining said aliquot with an aqueous suspension of a microparticulate carrier to form a clearing solution; and
   (b) separating the cells from the clearing solution.

2. The method of claim 1, wherein the microparticulate carrier is polystyrene beads 0.5 to 1.5 µm in diameter.

3. The method of claim 1 or claim 2, wherein the separating step comprises centrifuging the sample, to form a cell pellet.

4. The method of claim 3, comprising the additional step of removing the supernatant from the cell pellet, e.g. by aspirating the fluid above the pellet.

5. The method of claim 4, comprising the additional steps of adding a lysing agent to the cell pellet, to lyse the cells;

and testing the lysed sample to determine the relative amount of ATP which is present in the sample, e.g. by adding thereto a luciferase-luciferin reagent and measuring the relative light output emitted.

6. The method of any of claims 3 to 5, comprising, before centrifuging, the additional step of mixing with the aliquot a non-ionic detergent or other agent which lyses somatic cells in the sample but not microbial cells.

7. The method of any of claims 3 to 6, comprising the additional step of resuspending the pellet in a liquid and, if desired, testing the suspension to determine the concentration of microbial cells therein.

8. The method of claim 7, comprising the additional step of conducting a Breed Smear or other test on the resuspended pellet, to determine the cell titer.

9. The method of claim 7 or claim 8, wherein the liquid comprises a protease.

10. The method of any of claims 7 to 9, wherein the liquid comprises an agent which lyses somatic cells but not microbial cells.

11. The method of claim 10, comprising the additional steps of centrifuging the suspension, and separating the resultant second supernatant from the resultant second pellet which comprises primarily microbial cells.

12. The method of claim 11, comprising the additional step of testing the second supernatant for the concentration of ATP, e.g. by adding thereto a luciferase-luciferin reagent and measuring the relative light emitted.

13. The method of claim 11 or claim 12, comprising the additional steps of resuspending the second pellet in a liquid to give a second suspension, the liquid comprising an agent which lyses microbial cells or otherwise extracts ATP from microbial cells; and quantitatively testing the second suspension for ATP, e.g. by adding thereto a luciferase-luciferin reagent and measuring the relative amount of light emitted.

14. The method of claim 11, comprising the additional steps of resuspending the second pellet in a liquid to give a second suspension, the liquid comprising an agent which lyses somatic cells but not microbial cells; centrifuging the second suspension; removing the supernatant; and testing the resultant third pellet for the relative concentration of microbial cells.

15. The method of claim 14, comprising the additional step of resuspending the third pellet in a solution that stabilises the bacterial cells and prevents the loss of cellular metabolites.

16. The method of claim 15, wherein the stabilising solution contains magnesium ions and/or a protease.

17. A method of any preceding claim, wherein said material is milk, and the culture of said material is a liquid milk sample, and the method further comprises in step (a) mixing a chelating agent with the milk sample.

18. The method of claim 17, wherein the chelating agent is selected from bis(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, trans-1,2 diaminocyclohexan-etetraacetic acid, diethylenetriaminepentaacetic acid, citric acid, arginine, hypoxanthine, 4,5-dihydroxybenzene-1,3-disulfonic acid, sodium phosphate glass, crown ethertype compounds and derivatives and precursors thereof, and, more preferably, ethylenediaminetetraacetic acid or nitriloacetic acid.

19. The method of claim 17 or 18, wherein the separating step includes filtering the clearing solution with a filter which blocks cells and passes other milk components bound by the chelating agent.

20. A method-for detecting the presence of cells having nucleic acid that comprises a preselected target segment, in a culture or of an extract of a material of biological origin, the method comprising obtaining a pellet of cells from the culture or extract by using the method of claim 1 and using therein centrifugation, provided that, if said culture of a material of biological origin is a liquid milk sample, said clearing solution further comprises a chelating agent, e.g. as defined in claim 18; suspending cells from the pellet so obtained in a first solution and treating the first solution to provide a second solution of nucleic acid from the cells substantially without isolation of nucleic acids of the cells from other constituents of the cells; subjecting the nucleic acid of the second solution to a nucleic acid amplification process to provide a predetermined, amplified nucleic acid segment only if the preselected target

segment is present in said cells suspended from said pellet; and assaying nucleic acid after the amplification process for the presence of said predetermined, amplified segment.

21. The method of claim 20, wherein the amplification process is a PCR process, e.g. using as catalyst a Tag DNA polymerase.

22. The method of claim 20 or claim 21, wherein the cells to be detected are of Salmonella spp.

23. The method of any of claims 20 to 22, wherein said material is milk, and the culture of said material is a liquid milk sample.

24. The method of any preceding claim, wherein said material is a food material other than milk, e.g. a meat.

25. A clearing solution comprising an aliquot of a culture or of an extract of a material of biological origin, e.g. as defined in claim 23 or claim 24, and, in suspension, a microparticulate carrier, e.g. as defined in claim 2.

26. The solution of claim 25, which further comprises a chelating agent, e.g. as defined in claim 17, and a somatic cell-lysing agent.

27. The solution of claim 26, wherein the somatic cell-lysing agent is a non-ionic detergent.

28. A microbial test kit, for use in testing milk samples, comprising:

(i) the clearing solution of claim 26 or claim 27;
(ii) a solution comprising an ATP extractant which releases microbial cell ATP, e.g. an agent which lyses microbial cells; and
(iii) an ATP detection reagent, e.g. luciferin-luciferase.

29. The test kit of claim 28, wherein the ATP extractant is a trichloroacetic acid solution, optionally also containing ethylenediaminetetraacetic acid and xylenol blue.

30. A microbial test kit for testing milk samples in conjunction with a cell count test such as the Breed Smear, comprising:

(i) the clearing solution of claim 26 or claim 27; and
(ii) a solution for staining cells.

31. The test kit of any of claims 28 to 30, additionally including a solution for washing a cell pellet resulting from centrifugation, and, optionally, a buffer solution.

32. A test kit, for use in testing milk samples for somatic cells, comprising:

(i) a clearing solution comprising a chelating agent, e.g. as defined in claim 18, and, in suspension, a microparticulate carrier, e.g. as defined in claim 2;
(ii) a solution comprising an agent which lyses somatic cells but not microbial cells, e.g. as defined in claim 27; and
(iii) an ATP detection reagent, e.g. luciferin-luciferase.

33. The test kit of any of claims 28 to 32, further including a filter for filtering cells from milk components.

34. A test kit for the detection of cells, e.g. Salmonella spp, having nucleic acid that comprises a preselected target segment, the kit comprising a microparticulate carrier, e.g. as defined in claim 2; enzyme, e.g. Tag DNA polymerase, and primers or probes necessary for a nucleic acid amplification process to provide a predetermined, amplified nucleic acid segment only if the preselected target segment is present in the cells; and reagents required for assay of the amplified nucleic acid.

35. The kit of claim 34, for detection of cells in liquid milk samples, additionally comprising a chelating agent, e.g. as defined in claim 18.

Patentansprüche

1. Verfahren zur Abtrennung und Konzentrierung von Zellen aus einem Anteil einer Kultur oder eines Extrakts eines Materials mit biologischem Ursprung, das folgende Stufen umfaßt:

   (a) Kombination des Anteils mit einer wäßrigen Suspension eines mikroteilchenartigen Trägers unter Bildung einer Klärlösung; und
   (b) Abtrennung der Zellen aus der Klärlösung.

2. Verfahren nach Anspruch 1, wobei es sich bei dem mikroteilchenartigen Träger um Polystyrolkügelchen mit einem Durchmesser von 0,5 bis 1,5 µm handelt.

3. Verfahren nach Anspruch 1 oder 2, wobei die Trennstufe das Zentrifugieren der Probe unter Bildung eines Zellpellets umfaßt.

4. Verfahren nach Anspruch 3, das die zusätzliche Stufe der Entfernung des Überstands von dem Zellpellet, z. B. durch Absaugen der Flüssigkeit über dem Pellet, umfaßt.

5. Verfahren nach Anspruch 4, das die zusätzlichen Stufen der Zugabe eines lysierenden Mittels zu dem Zellpellet, um die Zellen zu lysieren; und des Tests der lysierten Probe zur Bestimmung der relativen Menge an ATP, die in der Probe vorhanden ist, z. B. durch Zugabe eines Luciferase-Luciferin-Reagenzes und Messung der emittierten relativen Lichtabgabe, umfaßt.

6. Verfahren nach einem der Ansprüche 3 bis 5, das vor dem Zentrifugieren die zusätzliche Stufe des Mischens eines nicht-ionischen Detergens oder eines anderen Mittels, das somatische Zellen in der Probe, nicht jedoch mikrobielle Zellen lysiert, mit dem Anteil umfaßt.

7. Verfahren nach einem der Ansprüche 3 bis 6, das die zusätzliche Stufe der Resuspendierung des Pellets in einer Flüssigkeit und gegebenenfalls des Tests der Suspension zur Bestimmung der Konzentration an mikrobiellen Zellen darin umfaßt.

8. Verfahren nach Anspruch 7, das die zusätzliche Stufe der Durchführung eines Breed-Ausstriches oder eines anderen Tests mit dem resuspendierten Pellet zur Bestimmung des Zelltiters umfaßt.

9. Verfahren nach Anspruch 7 oder 8, wobei die Flüssigkeit eine Protease enthält.

10. Verfahren nach einem der Ansprüche 7 bis 9, wobei die Flüssigkeit ein Mittel enthält, das somatische Zellen, nicht jedoch mikrobielle Zellen lysiert.

11. Verfahren nach Anspruch 10, das die zusätzlichen Stufen der Zentrifugation der Suspension und der Abtrennung des erhaltenen zweiten Überstands von dem erhaltenen zweiten Pellet, das hauptsächlich mikrobielle Zellen enthält, umfaßt.

12. Verfahren nach Anspruch 11, das die zusätzliche Stufe des Tests des zweiten Überstands auf die Konzentration an ATP, z. B. durch Zugabe eines Luciferase-Luciferin-Reagenzes und Messung des relativen emittierten Lichts, umfaßt.

13. Verfahren nach Anspruch 11 oder 12, das die zusätzlichen Stufen der Resuspendierung des zweiten Pellets in einer Flüssigkeit, wobei man eine zweite Suspension erhält und wobei die Flüssigkeit ein Mittel enthält, das mikrobielle Zellen lysiert oder auf andere Weise ATP aus mikrobiellen Zellen extrahiert; und den quantitativen Test der zweiten Suspension auf ATP, z. B. durch Zugabe eines Luciferase-Luciferin-Reagenzes und Messung der relativen Menge an emittiertem Licht, umfaßt.

14. Verfahren nach Anspruch 11, das die zusätzlichen Stufen der Resuspendierung des zweiten Pellets in einer Flüssigkeit, wobei man eine zweite Suspension erhält und wobei die Flüssigkeit ein Mittel enthält, das somatische Zellen, nicht jedoch mikrobielle Zellen lysiert; der Zentrifugation der zweiten Suspension; der Entfernung des Überstands; und des Tests des erhaltenen dritten Pellets auf die relative Konzentration an mikrobiellen Zellen umfaßt.

15. Verfahren nach Anspruch 14, das die zusätzliche Stufe der Resuspendierung des dritten Pellets in einer Lösung umfaßt, die die bakteriellen Zellen stabilisiert und einen Verlust an zellulären Metaboliten verhindert.

16. Verfahren nach Anspruch 15, wobei die Stabilisierungslösung Magnesiumionen und/oder eine Protease enthält.

17. Verfahren nach einem der vorstehenden Ansprüche, wobei es sich bei dem Material um Milch handelt und die Kultur des Materials eine flüssige Milchprobe ist und das Verfahren in Stufe (a) ferner das Mischen eines chelatisierenden Mittels mit der Milchprobe umfaßt.

18. Verfahren nach Anspruch 17, wobei das chelatisierende Mittel unter Bis-(o-aminophenoxy)-ethan-N,N,N',N'-tetraessigsäure, Ethylenglykol-bis-(β-aminoethylether)-N,N,N',N'-tetraessigsäure, trans-1,2-Diaminocyclohexantetraessigsäure, Diethylentriaminpentaessigsäure, Citronensäure, Arginin, Hypoxanthin, 4,5-Dihydroxybenzol-1,3-disulfonsäure, Natriumphosphatglas, Verbindungen vom Kronenethertyp sowie Derivaten und Vorstufen davon und insbesondere unter Ethylendiamintetraessigsäure oder Nitrilotriessigsäure ausgewählt ist.

19. Verfahren nach Anspruch 17 oder 18, wobei die Trennstufe das Filtrieren der Klärlösung mit einem Filter, der Zellen zurückhält und andere Milchkomponenten, die von dem chelatisierenden Mittel gebunden sind, durchläßt, umfaßt.

20. Verfahren zum Nachweis des Vorhandenseins von Zellen mit Nucleinsäure, die ein ausgewähltes Zielsegment umfaßt, in einer Kultur oder einem Extrakt eines Materials mit biologischem Ursprung, wobei das Verfahren es umfaßt, ein Pellet von Zellen aus der Kultur oder dem Extrakt unter Anwendung des Verfahrens von Anspruch 1 und unter Anwendung einer Zentrifugation bei diesem Verfahren zu erhalten, mit der Maßgabe, daß, wenn die Kultur eines Materials mit biologischem Ursprung eine flüssige Milchprobe ist, die Klärlösung ferner ein chelatisierendes Mittel, z. B. gemäß der Definition in Anspruch 18, enthält; die Zellen aus dem auf diese Weise erhaltenen Pellet in einer ersten Lösung zu suspendieren und die erste Lösung zur Bereitstellung einer zeiten Lösung von Nucleinsäure aus den Zellen im wesentlichen ohne Isolierung der Nucleinsäuren der Zellen von anderen Bestandteilen der Zellen zu behandeln; die Nucleinsäure der zweiten Lösung einem Nucleinsäure-Amplifikationsverfahren zur Bereitstellung eines festgelegten amplifizierten Nucleinsäuresegments, nur wenn das ausgewählte Zielsegment in den Zellen, die aus dem Pellet suspendiert wurden, enthalten ist, zu unterwerfen; und Nucleinsäure nach dem Amplifikationsverfahren auf das Vorhandensein des festgelegten amplifizierten Segments zu untersuchen.

21. Verfahren nach Anspruch 20, wobei das Amplifikationsverfahren ein PCR-Verfahren ist, z. B. unter Verwendung einer Taq-DNA-Polymerase als Katalysator.

22. Verfahren nach Anspruch 20 oder 21, wobei es sich bei den nachzuweisenden Zellen um Salmonella spp. handelt.

23. Verfahren nach Anspruch nach einem der Ansprüche 20 bis 22, wobei es sich bei dem Material um Milch handelt und die Kultur des Materials eine flüssige Milchprobe ist.

24. Verfahren nach Anspruch nach einem der vorstehenden Ansprüche, wobei es sich bei dem Material um ein Lebensmittelmaterial, das von Milch verschieden ist, z. B. Fleisch, handelt.

25. Klärlösung, die einen Anteil einer Kultur oder eines Extrakts eines Materials mit biologischem Ursprung, z.B. gemäß der Definition in Anspruch 23 oder 24, und in Suspension einen mikroneilchenartigen Träger, z. B. gemäß der Definition in Anspruch 2, enthält.

26. Lösung nach Anspruch 25, die ferner ein chelatisierendes Mittel, z. B. gemäß der Definition in Anspruch 17, und ein Somazellen-Lysemittel enthält.

27. Lösung nach Anspruch 26, wobei es sich bei dem Somazellen-Lysemittel um ein nicht-ionisches Detergens handelt.

28. Mikrobielle Testpackung für die Verwendung beim Testen von Milchproben, enthaltend:

(i) die Klärlösung nach Anspruch 26 oder 27;
(ii) eine Lösung, die ein ATP-Extraktionsmittel enthält, das mikrobielles Zell-ATP freisetzt, z. B. ein Mittel, das mikrobielle Zellen lysiert; und
(iii) ein ATP-Nachweisreagenz, z. B. Luciferin-Luciferase.

29. Testpackung nach Anspruch 28, wobei es sich bei dem ATP-Extraktionsmittel um eine Trichloressigsäurelösung, die wahlweise auch Ethylendiamintetraessigsäure und Xylenolblau enthält, handelt.

30. Mikrobielle Testpackung zum Testen von Milchproben im Zusammenhang mit einem Zellzähltest, wie dem Breed-Ausstrich, umfassend:

(i) die Klärlösung nach Anspruch 26 oder 27; und
(ii) eine Lösung zum Anfärben von Zellen.

31. Testpackung nach einem der Ansprüche 28 bis 30, die zusätzlich eine Lösung zum Waschen eines Zellpellets, das bei der Zentrifugation erhalten wird, und wahlweise eine Pufferlösung enthält.

32. Testpackung zur Verwendung beim Testen von Milchproben auf somatische Zellen, umfassend:

(i) eine Klärlösung, die ein chelatisierendes Mittel, z. B. gemäß der Definition in Anspruch 18, und in Suspension einen mikroteilchenartigen Träger, z. B. gemäß der Definition in Anspruch 2, enthält;
(ii) eine Lösung, die ein Mittel enthält, das somatische Zellen, nicht jedoch mikrobielle Zellen lysiert, z. B. gemäß der Definition in Anspruch 27; und
(iii) ein ATP-Nachweisreagenz, z. B. Luciferin-Luciferase.

33. Testpackung nach einem der Ansprüche 28 bis 32, die ferner einen Filter zum Abfiltrieren von Zellen von Milchbestandteilen umfaßt.

34. Testpackung zum Nachweis von Zellen, z. B. Salmonella spp., mit Nucleinsäure, die ein ausgewähltes Zielsegment umfaßt, wobei die Testpackung einen mikroteilchenartigen Träger, z. B. gemäß der Definition in Anspruch 2; Enzym, z. B. Taq-DNA-Polymerase, und Primer oder Sonden, die für ein Nucleinsäure-Amplifikationsverfahren zur Bereitstellung eines festgelegten amplifizierten Nucleinsäuresegments, nur wenn das ausgewählte Zielsegment in den Zellen vorhanden ist; und Reagenzien, die für eine Untersuchung der amplifizierten Nucleinsäure erforderlich sind, umfaßt.

35. Testpackung nach Anspruch 34 zum Nachweis von Zellen in flüssigen Milchproben, die zusätzlich ein chelatisierendes Mittel, z. B. gemäß der Definition in Anspruch 18, umfaßt.

Revendications

1. Procédé de séparation et de concentration de cellules à partir d'une quantité aliquote d'une culture ou d'un extrait d'une substance d'origine biologique, comprenant les étapes consistant à:

(a) combiner ladite quantité aliquote avec une suspension aqueuse d'un support microparticulaire pour former une solution de clarification et
(b) séparer les cellules de la solution de clarification.

2. Procédé selon la revendication 1, où le support microparticulaire est constitué par des billes de polystyrène de 0,5 à 1,5 µm de diamètre.

3. Procédé selon la revendication 1 ou la revendication 2, où l'étape de séparation comprend la centrifugation de l'échantillon, pour former un culot cellulaire.

4. Procédé selon la revendication 3, comprenant l'étape additionnelle consistant à séparer le surnageant du culot cellulaire, par exemple par aspiration du fluide se trouvant au-dessus du culot.

5. Procédé selon la revendication 4, comprenant les étapes additionnelles consistant à ajouter un agent de lyse au culot cellulaire, pour lyser les cellules, et à tester l'échantillon lysé pour déterminer la quantité relative d'ATP présente dans l'échantillon, par exemple par addition d'un réactif luciférase-luciférine et mesure de la puissance lumineuse relative émise.

6. Procédé selon l'une quelconque des revendications 3 à 5, comprenant, avant la centrifugation, l'étape additionnelle

consistant à mélanger à la quantité aliquote un détergent non ionique ou autre agent qui lyse les cellules somatiques dans l'échantillon, mais non les cellules microbiennes.

7. Procédé selon l'une quelconque des revendications 3 à 6, comprenant l'étape additionnelle consistant à remettre le culot en suspension dans un liquide et, si désiré, à doser la suspension pour déterminer la concentration de cellules microbiennes qu'elle contient.

8. Procédé selon la revendication 7, comprenant l'étape additionnelle consistant à procéder à un "Breed Smear" ou autre dosage sur le culot remis en suspension, pour déterminer le titre cellulaire.

9. Procédé selon la revendication 7 ou la revendication 8, où le liquide comprend une protéase.

10. Procédé selon l'une quelconque des revendications 7 à 9, où le liquide comprend un agent qui lyse les cellules somatiques mais non les cellules microbiennes.

11. Procédé selon la revendication 10, comprenant les étapes additionnelles consistant à centrifuger la suspension et à séparer le deuxième surnageant obtenu du deuxième culot obtenu, lequel comprend essentiellement des cellules microbiennes.

12. Procédé selon la revendication 11, comprenant l'étape additionnelle consistant à déterminer la concentration d'ATP dans le deuxième sumageant, par exemple par addition d'un réactif luciférase-luciférine et mesure de la lumière relative émise.

13. Procédé selon la revendication 11 ou la revendication 12, comprenant les étapes additionnelles consistant à remettre le deuxième culot en suspension dans un liquide pour obtenir une deuxième suspension, le liquide comprenant un agent qui lyse les cellules microbiennes ou extrait d'autre façon l'ATP de cellules microbiennes ; et à déterminer quantitativement l'ATP dans la deuxième suspension, par exemple en ajoutant un réactif luciférase-luciférine et en mesurant la quantité relative de lumière émise.

14. Procédé selon la revendication11, comprenant les étapes additionnelles consistant à remettre le deuxième culot en suspension dans un liquide pour obtenir une deuxième suspension, le liquide comprenant un agent qui lyse les cellules somatiques mais non les cellules microbiennes ; à centrifuger la deuxième suspension ; à séparer le surnageant ; et à déterminer la concentration relative de cellules microbiennes dans le troisième culot obtenu.

15. Procédé selon la revendication 14, comprenant l'étape additionnelle consistant à remettre le troisième culot en suspension dans une solution qui stabilise les cellules bactériennes et empêche la perte de métabolites cellulaires.

16. Procédé selon la revendication 15, où la solution stabilisante contient des ions magnésium et/ou une protéase.

17. Procédé selon l'une quelconque des revendications précédentes, où ladite substance est du lait et la culture de ladite substance est un échantillon de lait liquide et le procédé comprend en outre à l'étape (a) le mélange d'un agent chélatant avec l'échantillon de lait.

18. Procédé selon la revendication 17, où l'agent chélatant est sélectionné parmi l'acide bis(o-aminophénoxy)-éthane-N,N,N',N'-tétraacétique, l'acide éthylèneglycol-bis(β-aminoéthyléther)-N,N,N',N'-tétraacétique, l'acide trans-1,2-diaminocyclohexanetétraacétique, l'acide diéthylènetriaminopentaacétique, l'acide citrique, l'arginine, l'hypoxanthine, l'acide 4,5-dihydroxybenzène-1,3-disulfonique, le verre au phosphate de sodium, les composés de type éthers-couronnes et leurs dérivés et leurs précurseurs, et de façon particulièrement préférée, l'acide éthylènediaminotétraacétique ou l'acide nitriloacétique.

19. Procédé selon la revendication 17 ou 18, où l'étape de séparation comprend la filtration de la solution de clarification au moyen d'un filtre qui arrête les cellules et laisse passer les autres composants du lait liés par l'agent chélatant.

20. Procédé de détection de la présence de cellules ayant un acide nucléique qui comprend un segment cible présélectionné, dans une culture ou un extrait d'une substance d'origine biologique, le procédé comprenant l'obtention d'un culot de cellules à partir de la culture ou de l'extrait par recours au procédé selon la revendication 1 et utilisation dans ce cas de la centrifugation, pour autant que, lorsque ladite culture d'une substance d'origine biologique est un échantillon de lait liquide, ladite solution de clarification comprend en outre un agent chélatant, par exemple

tel que défini à la revendication 18 ; la mise en suspension de cellules du culot ainsi obtenu dans une première solution et le traitement de la première solution pour obtenir une deuxième solution d'acide nucléique des cellules sensiblement sans isoler les acides nucléiques des cellules des autres constituants cellulaires ; la soumission de l'acide nucléique de la deuxième solution à un processus d'amplification d'acide nucléique pour n'obtenir un segment d'acide nucléique amplifié prédéterminé que si le segment cible présélectionné est présent dans lesdites cellules mises en suspension à partir dudit culot ; et le dosage de l'acide nucléique après le processus d'amplification pour déterminer la présence dudit segment amplifié prédéterminé.

21. Procédé selon la revendication 20, où le processus d'amplification est un processus de PCR, utilisant par exemple comme catalyseur une Tag ADN-polymérase.

22. Procédé selon la revendication 20 ou la revendication 21, où les cellules à détecter sont des cellules de Salmonella spp.

23. Procédé selon l'une quelconque des revendications 20 à 22, où ladite substance est du lait et la culture de ladite substance est un échantillon de lait liquide.

24. Procédé selon l'une quelconque des revendications précédentes, où ladite substance est une denrée alimentaire autre que le lait, par exemple de la viande.

25. Solution de clarification comprenant une quantité aliquote d'une culture ou d'un extrait d'une substance d'origine biologique, par exemple telle que définie à la revendication 23 ou la revendication 24, et, en suspension, un support microparticulaire, par exemple tel que défini à la revendication 2.

26. Solution selon la revendication 25, laquelle comprend en outre un agent chélatant, par exemple tel que défini à la revendication 17, et un agent de lyse des cellules somatiques.

27. Solution selon la revendication 26, où l'agent de lyse des cellules somatiques est un détergent non ionique.

28. Kit de dosage microbien, à utiliser dans le dosage d'échantillons de lait, comprenant :

(i) la solution de clarification selon la revendication 26 ou la revendication 27 ;
(ii) une solution comprenant un extracteur d'ATP qui libère l'ATP des cellules microbiennes, par exemple un agent qui lyse les cellules microbiennes ; et
(iii) un réactif de détection de l'ATP, par exemple luciférine-luciférase.

29. Kit de dosage selon la revendication 28, où l'extracteur d'ATP est une solution d'acide trichloroacétique, contenant également facultativement de l'acide éthylènediaminotétraacétique et du bleu xylénol.

30. Kit de dosage microbien destiné au dosage d'échantillons de lait associé à un dosage de numération cellulaire tel que le "Breed Smear", comprenant:

(i) la solution de clarification selon la revendication 26 ou la revendication 27 ; et
(ii) une solution de coloration de cellules.

31. Kit de dosage selon l'une quelconque des revendications 28 à 30, comprenant additionnellement une solution de lavage d'un culot cellulaire résultant de la centrifugation et, facultativement, une solution tampon.

32. Kit de dosage à utiliser dans le dosage de cellules somatiques dans des échantillons de lait, comprenant :

(i) une solution de clarification comprenant un agent chélatant, par exemple tel que défini selon la revendication 18, et, en suspension, un support microparticulaire, par exemple tel que défini selon la revendication 2 ;
(ii) une solution comprenant un agent qui lyse les cellules somatiques mais non les cellules microbiennes, par exemple tel que défini selon la revendication 27 ; et
(iii) un réactif de détection de l'ATP, par exemple luciférine-luciférase

33. Kit de dosage selon l'une quelconque des revendications 28 à 32, comprenant en outre un filtre destiné à séparer les cellules des composants du lait.

34. Kit de dosage destiné à la détection de cellules, par exemple Salmonella spp., ayant un acide nucléique qui comprend un segment cible présélectionné, le kit comprenant un support microparticulaire, par exemple tel que défini selon la revendication 2 ; une enzyme, par exemple Taq ADN-polymérase, et des amorces ou sondes nécessaires pour un processus d'amplification d'acide nucléique, pour n'obtenir un segment d'acide nucléique amplifié prédéterminé que si le segment cible présélectionné est présent dans les cellules ; et des réactifs requis pour détecter l'acide nucléique amplifié.

35. Kit selon la revendication 34, destiné à la détection de cellules dans des échantillons de lait liquide, comprenant additionnellement un agent chélatant, par exemple tel que défini selon la revendication 18.

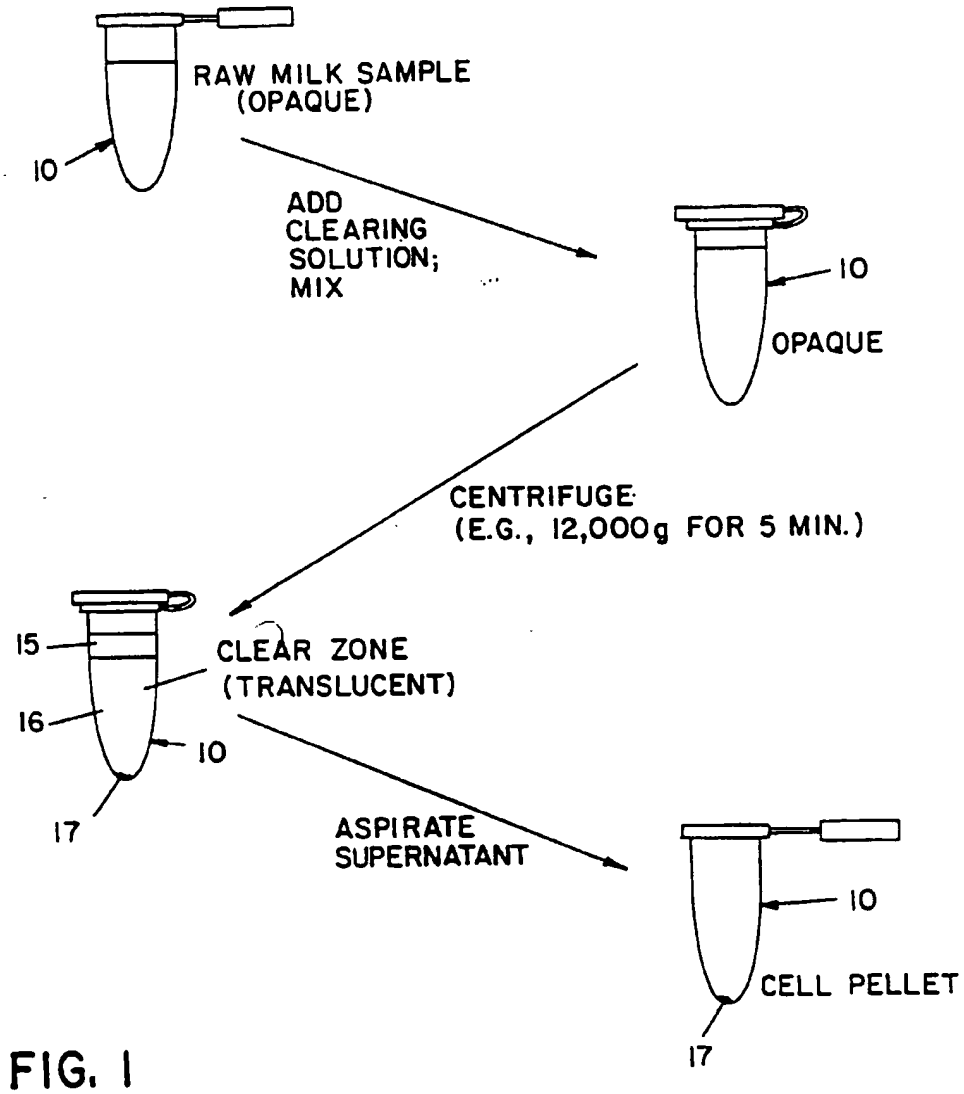
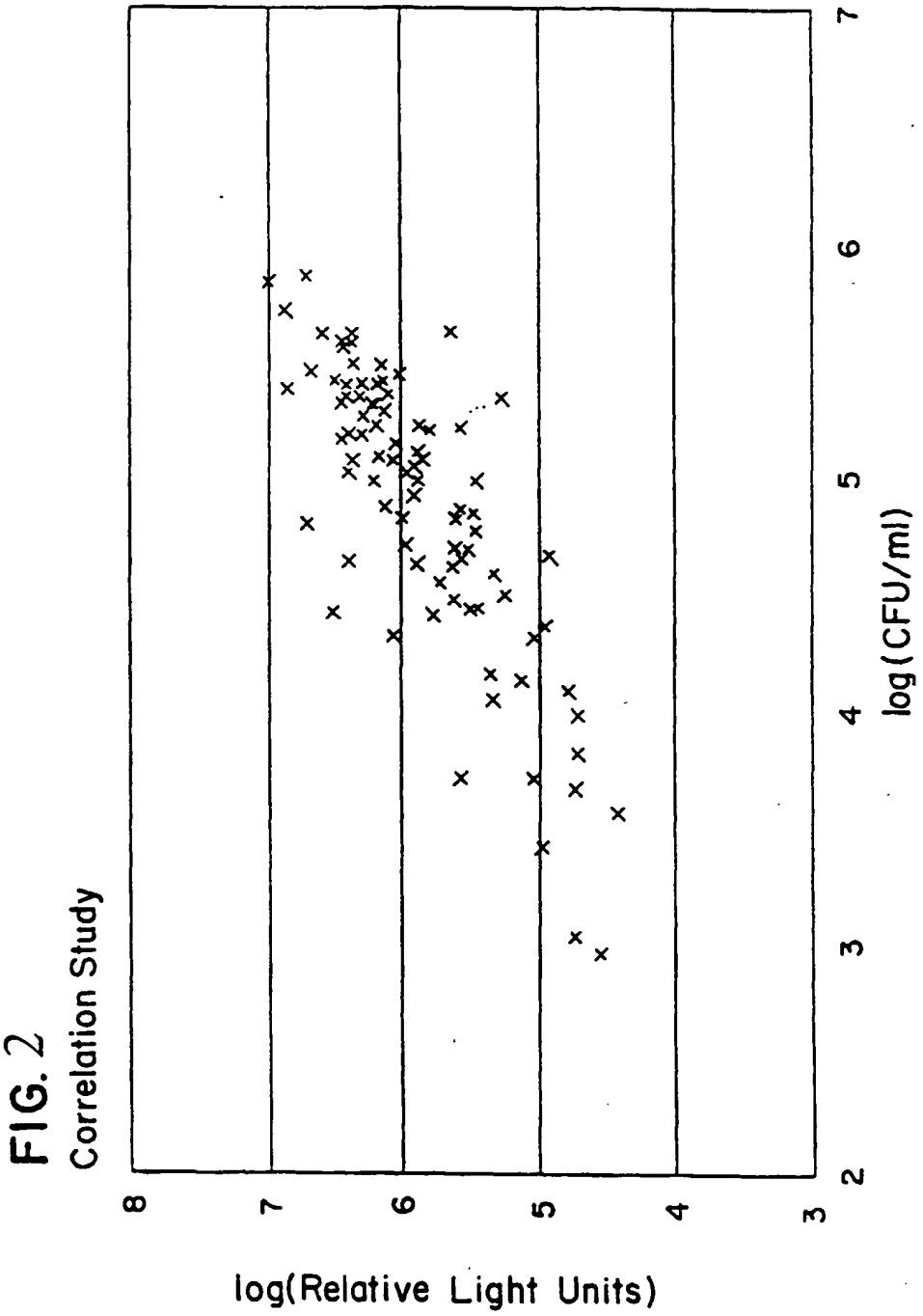


FIG. 1


Correlation Study
log(CFU/ml)
log(Relative Light Units)
8
7
6
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4
3
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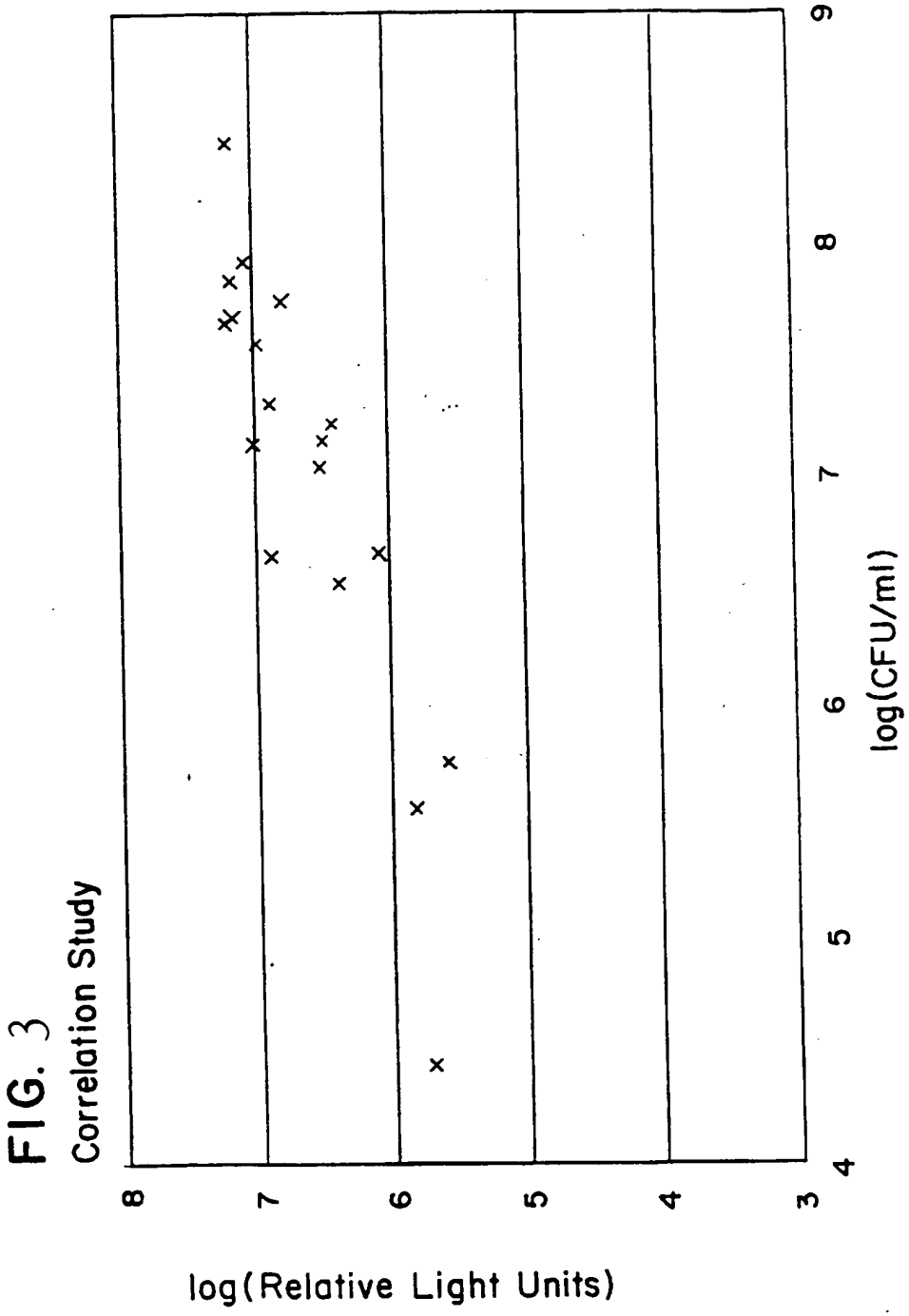
FIG. 2


Correlation Study
log(CFU/ml)
9
8
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3
log(Relative Light Units)

FIG. 3